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Intraglomerular platelet aggregation and experimental glomerulonephritis

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Intraglomerular platelet aggregation and experimental glomerulonephritis. Oxygen free radical production inhibits ADPase-mediated antithrombotic action. Different forms of experimental glomerulonephritis (GN) are characterized by early glomerular influx of inflammatory cells and thrombus formation. The causal relationship of these inflammatory events is obscure. Previous studies have shown that glomerular ADPase in the rat kidney may function as a potent antithrombotic principle, whereas this enzyme is highly sensitive for oxygen free radicals. To study whether O_2^- producing inflammatory cells are able to induce intraglomerular thrombosis via impairment of ADPase, we investigated influx of inflammatory cells in relation to glomerular ADPase activity and platelet aggregation in three models of GN. In two of these models (anti-Thy1 and anti-GBM GN) influx of neutrophils and thrombus formation occurs, whereas in anti-FX1A nephritis this aspect of the inflammatory phase is not present. The results show a relationship between influx of oxygen free radical-producing cells, reduction of glomerular ADPase activity and increased platelet aggregation. Moreover, it is shown that impairment of glomerular ADPase and increased platelet aggregation in anti-Thy1 and anti-GBM GN could be reduced by treatment with superoxide dismutase and catalase. The demonstration that activated neutrophils perfused ex vivo in the rat kidney can directly affect glomerular ADPase and antithrombotic potential in an O_2^- dependent manner, further supports the proposed sequence of events; oxygen free radicals produced by activated neutrophils reduce glomerular ADPase activity, leading to facilitation of thrombus formation.

Inflammation, both in clinical as well as in experimental conditions is often associated with platelet aggregation [1, 2]. In different forms of experimental acute glomerulonephritis (GN) such as nephrotoxic serum nephritis or GN induced by monoclonal anti-Thy1 antibodies (referred to as anti-Thy1 nephritis) [3] influx of neutrophils can be observed in glomeruli [4–6], and intraglomerular platelet aggregation is also a common phenomenon [4, 7]. However, the causal relationship of these intraglomerular inflammatory events is obscure.

Platelet aggregation can be initiated in several ways during the process of inflammation. For instance, it has been shown that thromboxane A_2 (TXA_2) production is increased in glomeruli of rats with nephrotoxic serum nephritis [8]. Other possibilities include platelet activation by immune complexes via their Fc receptors [9] or by increased synthesis of platelet activating factor (PAF) during the inflammatory condition [7, 10]. In addition, already existing antithrombotic mechanisms, that is, vessel wall-associated ADPase [11, 12], may be affected in acute inflammation. Since recently this antithrombotic principle has been suggested to be of major importance in preventing experimental intraglomerular thrombus formation in the rat kidney [13], we investigated the influence of inflammatory cells upon this specific antithrombotic mechanism in the rat kidney in vivo and ex vivo. The implication of glomerular ADPase seems particularly relevant since neutrophils produce excess of oxygen free radicals after activation ("respiratory burst") [14], whereas these membrane associated enzymes are highly susceptible for toxic oxygen products [15–17].

Therefore, we studied glomerular inflammatory cell infiltration in relation with ADPase activity and platelet aggregation during the early phase of three forms of experimental GN in rats with and without treatment with oxygen radical scavengers. In two of these experimental models (anti-GBM and anti-Thy1) acute inflammation with influx of polymorphonuclear neutrophils (PMN) and thrombus formation occurs [4, 7], whereas in passive Heymann glomerulopathy induced by heterologous anti-FX1A IgG this aspect of the inflammatory phase is not present [18, 19]. Since immune complex formation obviously occurs in glomeruli during anti-FX1A nephritis, this model is appropriate as a control model to study the role of antibody deposition *per se* upon glomerular ADPase and platelet activation.

The results show a relationship between the magnitude of PMN infiltration and the reduction of glomerular ADPase activity on the one hand and increased intraglomerular platelet aggregation on the other hand. Moreover, it appears that ADPase impairment and increased thrombotic tendency induced by activated PMN can be prevented by superoxide dismutase (SOD) and catalase.

These studies confirm the importance of intraglomerular ADP-ase activity as an antithrombotic mechanism in the rat kidney. In addition a new mechanism is suggested in which activated PMN may promote intraglomerular platelet aggregation in anti-Thy1 and anti-GBM nephritis in the rat.

Methods

Experimental animals

Female inbred PVG/c rats, two months of age, fed ad libitum with standard chow (Hope Farms, Woerden, The Netherlands), were used throughout the study.

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In vivo studies

Induction of experimental glomerulonephritis. Anti-Thy1 nephritis was induced by a single i.v. injection of mouse IgG against rat Thy1 in a dosis of 5 mg/kg body wt [3]. Anti-GBM glomerulonephritis was induced by an i.v. injection of 0.5 ml heterologous rabbit antiserum against rat glomerular basement membrane (GBM), provided by Dr. E. de Heer (Leiden, The Netherlands). Rabbit anti-FX1A IgG was prepared according to standard procedures using tubular brush border antigens of rat kidneys [19]. Rats received a single i.v. injection of 5 mg/kg body wt. IgG after absorption of this IgG to isolated GBM fractions according to standard procedures [19].

Treatment with oxygen radical scavengers. Part of the animals with anti-Thy1 or anti-GBM nephritis received a treatment with both SOD (3333 U/mg protein, Serva, Heidelberg, FRG) and catalase (3800 U/mg protein, Sigma Chemical Co, St. Louis, Missouri, USA). Immediately after induction of the disease the mixture of SOD and catalase was i.v. administered followed by i.p. injections after 4, 16 and 20 hours. Rats received 5 mg/kg body wt SOD and 60 mg/kg body wt catalase per injection.

Ex vivo perfusion studies

Cell preparations. To study the influence of activated inflammatory cells upon glomerular ADPase activity and thrombotic tendency, kidney perfusions ex vivo were performed with activated rat peritoneal exsudate cells (PEC) and human platelet-rich plasma (PRP).

PEC suspensions were obtained from the peritoneal cavity of rats 15 hours after an i.p. injection of 7 ml saline with 10% proteose pepton (Difco) and 0.8% heart infusion broth (Difco) [20]. PEC were harvested by washing the peritoneal cavity with Mg^{++}, Ca^{++} free Hank's balanced salt solution (HBSS; pH 7.2) at 4°C. Cell suspensions were washed twice with HBSS by centrifugation (10 min 200 × g) and volumes were adjusted to a cell concentration of 7.0×10^5 cells/ml. Purity of the cell suspension was checked by morphological criteria (approximately 90% PMN and 10% monocytes). Suspension were kept at 4°C until used.

PRP obtained from citrated blood, drawn from healthy volunteers and prepared according to standard procedures [13] was kept in polytefin (PL 732) bags in a shaker to prevent aggregation, and stored at room temperature for not more than five days. Before perfusion PRP was centrifuged at 1000 × g for 15 minutes, and the platelet number was standardized to 2.7×10^9 cells/ml. Only platelet suspensions showing standard aggregation curves following ADP or ristocitin stimulation [21], as tested using an aggregometer (Biodata Corporation, model PAP-4) were used.

Kidney perfusion procedure ex vivo. Perfusion of the left kidney was performed according to the method of Hoyer, Mauer and Michael [22]. After removal of the blood with 4 ml phosphate buffered saline (PBS, pH 7.2 at 37°C), 10 ml PEC suspension (37°C) with or without 300 U/ml SOD (Serva) was perfused through the kidney using a perfusion pump (flow rate 2 ml/min). Kidneys of control rats were perfused with 10 ml HBSS and SOD (300 U/ml). Immediately after perfusion a biopsy was taken and snap frozen at −80°C. Subsequently the kidney was perfused with successively 1 ml ADP (Sigma, 10 μg/ml) in saline, 4 ml human PRP, 1 ml ADP (10 μg/ml), and 4 ml PRP (flow rate 2 ml/min). After perfusion kidney specimens were embedded in plastic (**Histochemistry**).

Histochemistry

Tissue processing. Kidney specimens were after removal immediately snap frozen in freon (−80°C). Tissue obtained from kidneys after alternate perfusion with platelets and ADP were embedded in glycolmetacrylate (60%)/butanediol monoacrylate (40%) plastic according to the method of van Goor et al [23]. Sections were studied for intraglomerular antibody deposition, the presence of activated neutrophils, ADPase activity and platelet aggregation.

Intraglomerular antibody deposition. Four micrometer thick kidney cryostat sections obtained from animals injected with anti-Thy1 IgG were stained with FITC-conjugated rabbit-anti-mouse IgG (Nordic, Tilburg, The Netherlands) according to standard procedures. Kidney sections from animals injected with anti-GBM serum and anti-FX1A IgG were stained with FITC-conjugated goat-anti-rabbit IgG (Nordic). Sections were examined with a Leitz Orthoplan fluorescence microscope (E. Leitz Inc., Rockleigh, New Jersey, USA).

Demonstration of activated neutrophils. Presence of neutrophils was demonstrated in air-dried, 4 μm cryostat sections by demonstrating peroxidase (PO) activity, which is shown to be quantitatively related to the number of neutrophils [24], taking advantage of endogenous H_2O_2 production by activated neutrophils [14]. Sections were incubated for 20 minutes at 37°C in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.5 mg/ml 3,3-diamino benzidine (DAB; Sigma Chemical Co). H_2O_2 production by neutrophils and concomitant PO activity will induce oxidation and polymerization of DAB. Addition of catalase to the incubation medium (0.01 to 0.1 mg/ml) inhibited staining in a dose dependent manner, confirming the involvement of endogenous H_2O_2 production in DAB polymerization. Superoxide anion production by activated PMN was demonstrated in kidney cryostat sections according to the method of Briggs et al [25], applied at the light microscopical level. This method is based upon the oxidation of Mn^{2+} to Mn^{3+} by O_2^- and the subsequent oxidation of DAB by Mn^{3+}. Inhibition of staining by addition of SOD to the incubation media (300 U/ml) confirms the demonstration of O_2^- production by this method. Sections were routinely stained with hematoxilin, and infiltrating cells were evaluated by morphological criteria.

ADPase activity. Enzyme activity was histochemically demonstrated at the light microscopical level using the cerium-based method [26, 27] with minor modifications. Briefly, formalin-macrodex fixed cryostat sections (4 μm) were preincubated for 15 minutes in 70 mM Tris-maleate buffer (pH 7.2) with 1 mM $CeCl_3$ and 5 mM $Mg(NO_3)_2$ to allow diffusion of cerium ions to the site of enzyme activity, followed by incubation in the same medium with 2.3 mM ADP (30 min, 37°C). To demonstrate reaction product at the light microscopical level cerium phosphate was converted to cerium perhydroxide [28] in glycine-NaOH buffer (pH 8.5) with 0.5% H_2O_2 during 20 minutes at room temperature. Subsequently, reaction product was contrasted with 0.5 mg/ml DAB in 0.1 M Tris/HCl (pH 7.6) at 60°C for 20 minutes [29, 30]. A dark staining of DAB indicated the presence of ADPase activity. No reaction product was ob-

served after incubations without ADP, which is in agreement with other studies [30].

Platelet aggregation. Intraglomerular platelet aggregation in different forms of nephritis was demonstrated in 4 μm cryostat sections using a mouse monoclonal antibody (Moab) against rat platelets (pl-1) [31]. The antibody was labeled with biotin (Dakopatts). After formalin-macrodex fixation, cryostat sections were preincubated with avidine (Sigma, 30 min) and biotin (30 min) and subsequently incubated with biotinilated pl-1 for 30 minutes at 37°C. Sections were incubated with avidin-biotin complex (Dakopatts, Glostrup, Denmark), and finally this complex was demonstrated with DAB and H_2O_2.

After ex vivo perfusion with PEC suspension or buffer (group IV, V and VI), glomerular thrombotic tendency was determined by demonstrating fibrinogen receptor exposure of human platelets perfused through the kidney. It has been shown that activated platelets expose membrane receptors for fibrinogen [32] leading to binding of fibrinogen [33]. Since citrated plasma was used, conversion of fibrinogen to fibrin was inhibited. Fibrinogen was demonstrated in 2 μm plastic sections by applying indirect immunoperoxidase methods with rabbit-anti-human fibrinogen (Behringwerke AG, Marburg, FRG) [23]. Endogenous peroxidase activity was inhibited in a methanol (30%)-H_2O_2(0.5%) mixture. Peroxidase conjugated swine-anti-rabbit IgG (Dakopatts) with 5% normal rat serum was used in the second step, and subsequently demonstrated with DAB.

Experimental design

To study intraglomerular platelet aggregation and ADPase activity in vivo, rats were divided in three groups receiving a single injection of either anti-Thy1 (group I, $N = 12$), anti-GBM (group II, $N = 12$) or anti-FX1A antibody (group III, $N = 6$). Control animals (group IV, $N = 6$) received an i.v. injection of saline. Six animals out of group I and II received treatment with SOD and catalase and six animals received treatment with saline.

Animals were sacrificed 24 hours after induction of the disease and kidneys were processed for histochemistry as decribed.

To study the direct effects of activated neutrophils upon glomerular ADPase activity and thrombotic tendency, left kidneys of normal rats were prepared for perfusion under halothane, N_2O/O_2 anaesthesia, receiving either PEC suspensions (group V; $N = 5$) or PEC supplemented with 300 U/ml SOD (group VI; $N = 5$). The control group received kidney perfusion with buffer supplemented with SOD without cells (group VII; $N = 5$), since it has been demonstrated that SOD *per se* does not influence glomerular ADPase activity [16]. Thrombotic tendency was subsequently assessed by alternate perfusion with PRP and ADP.

Care was taken that in parallel experiments the same batch of cells (PEC or PRP) were employed.

Quantitative analysis of tissue sections and statistical evaluation

To quantify the inflammatory response in different experimental animals the number of oxygen free radical-producing cells in glomeruli were counted. Presence of these cells was evaluated in cryostat sections by scoring the number of DAB positive spots per glomerulus after staining for peroxidase activity in 50 glomeruli per rat.

Glomerular ADPase activity and platelet aggregation in vivo detected by staining with Moab pl-1 were quantitatively evaluated at the light microscopical level by television-based image analyzing techniques. This analysis is based upon measuring light absorption by DAB precipitations in glomeruli, correlating with the degree of platelet aggregation or ADPase activity. The microscopical field was digitized by a frame grabber (PC-Plus, Imaging Tech.) in 256 × 256 pixels with possible grey levels from 0 to 255. Glomeruli were selected by using a digitizing tablet (mm1812, Summagraphics) and number of pixels above a threshold grey value were counted. Degree of platelet aggregation was expressed as mean percentage of glomerular area with DAB precipitation. Amount of reaction product after staining for ADPase activity was expressed in units, that is, average optical density (= log intensity) per standard glomerular area. The mean optical density or percentage platelet aggregation of twenty glomeruli per individual rat was assayed, and results were expressed as arithmetic means (± SD) of six animals per group.

Results of ex vivo perfusion studies were semiquantitatively evaluated in a double-blind scoring procedure, using an arbitrary scale by counting the relative number of glomeruli with normal versus reduced ADPase activity in kidney sections after PEC perfusion. Similarly, the number of glomeruli positive for human fibrinogen were scored after alternate perfusion with platelets and ADP. Fifty glomeruli per section were evaluated and results are expressed as mean percentage of positive glomeruli per section (± SD). Data were analyzed by Wilcoxon's test and were considered significant at $P < 0.05$.

Results

Experimental models of glomerulonephritis

Kidney sections of each individual rat injected with anti-Thy1 IgG showed typical mesangial fluorescence pattern when incubated with FITC-conjugated rabbit-anti-mouse IgG (group I). Neither staining pattern nor staining intensity was altered in animals receiving either saline or SOD and catalase after the induction of the disease. Kidney sections of rats with anti-GBM nephritis (group II) showed typical linear staining patterns along the glomerular capillary wall in all cases after incubation with FITC-conjugated goat-anti-rabbit IgG. Again, no differences in fluorescence pattern or intensity were observed between animals receiving treatment with scavengers as compared to animals injected with saline after induction of the disease. Twenty-four hours after injection with rabbit anti-FX1A IgG, an identical pattern of fine granular fluorescence along glomerular capillary walls in kidneys of each rat of this group was observed.

Influx of inflammatory cells

To examine the influx of inflammatory cells in glomeruli of rats with different forms of experimental GN, quantitative analysis of neutrophil infiltration was performed upon cryostat sections stained for endogenous peroxidase activity and concomitant H_2O_2 production with DAB as described in **Methods**. Identical staining patterns were observed when sections were stained for O_2^- producing cells with DAB (Fig. 1). As can be

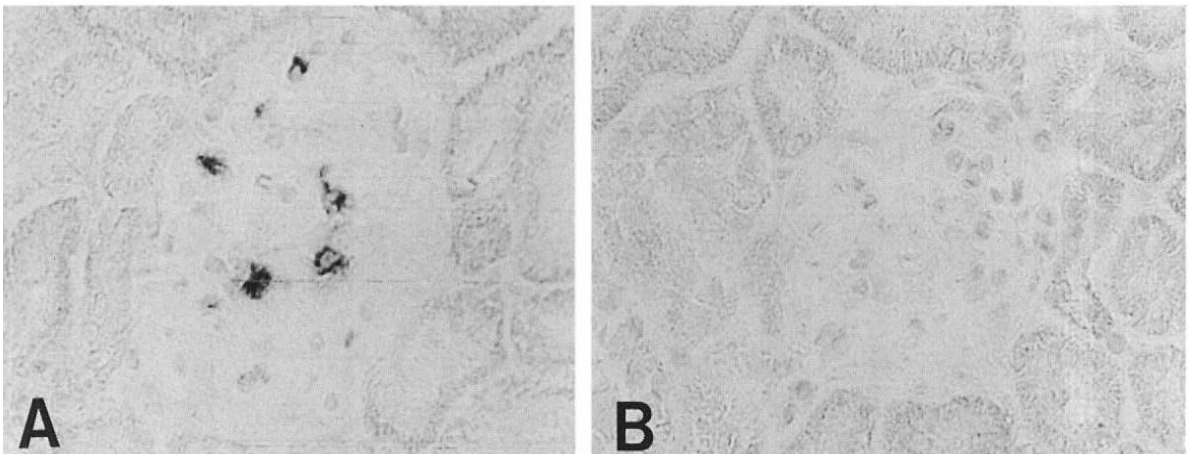

Fig. 1. *Staining for O_2^- activity in cryostat sections from kidney 24 hr after induction of* **(A)** *anti-Thy1 nephritis (group I) or* **(B)** *saline treated control rats (group IV).* Black dots in glomerulus of nephritic animal (A) represent DAB reaction product at the site of O_2^- production by inflammatory cells (original magnification ×350).

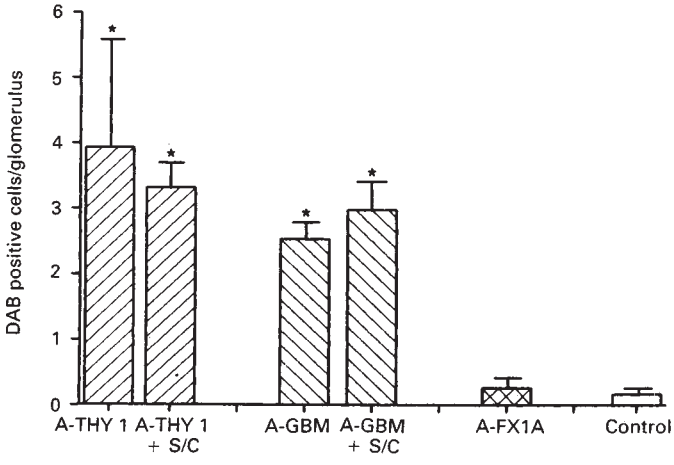

Fig. 2. *Analysis of the number of H_2O_2 producing cells in glomeruli of rats with different forms of glomerulonephritis (anti-Thy1, anti-GBM, anti-FX1A) or saline injected control rats receiving treatment with saline or SOD and catalase as indicated (+ S/C).* Columns represent arithmetic means (± SD) of DAB positive cells per glomerulus. Significant increase in number of cells can be observed in glomeruli of rats with anti-Thy1 (group I) and anti-GBM (group II) (hatched columns) compared to control rats (group IV) (open columns), whereas this cell number is not significantly influenced by treatment with SOD and catalase in vivo (hatched columns + S/C). * = statistical significance compared to control animals (open columns) as indicated, $P < 0.005$ (Wilcoxon). Abbreviations are: A-Thy1, anti-Thy1 glomerulonephritis; A-GBM, anti-GBM glomerulonephritis; A-FX1A, anti-FX1A glomerulonephritis; contr, saline injected rats; S/C, treatment with SOD and catalase.

seen in Figure 2, a significant increase in DAB positive cells was demonstrated in glomeruli of rats with anti-Thy1 (group I) and anti-GBM (group II) nephritis (3.92 ± 1.6 and 2.52 ± 0.5 cells/glomerulus, respectively) as compared to saline injected control rats (0.3 ± 0.2 cells/glomerulus, group IV, $P < 0.005$). In rats with anti-FX1A nephritis (group III) no significant increase in mean number of DAB positive cells could be observed (0.3 ± 0.1 cells/glomerulus) as compared to control rats (group IV). In the SOD and catalase treated rats no change in the number of PMN per glomerulus in the anti-Thy1 nor in the anti-GBM nephritic rats was observed (3.3 ± 0.4 and 2.9 ± 0.5 cells/glomerulus, respectively) as compared to saline-treated nephritic rats.

ADPase activity

Reduction in glomerular ADPase activity could be demonstrated at the light microscopical level in glomeruli of rats with anti-Thy1 (group I) and anti-GBM nephritis (group II) as compared to glomeruli of control rats (group IV, Fig. 3). In contrast, no reduction in glomerular ADPase activity was observed in animals with anti-FX1A nephritis (group III).

Quantitative analysis of glomerular ADPase activity confirms these observations (Fig. 4). Saline injected rats (group IV) showed an average glomerular ADPase activity of 62.9 ± 14.8 units, whereas in animals with anti-Thy1 nephritis (group I) ADPase activity was significantly reduced (13.5 ± 4.6 units, $P < 0.005$). ADPase activity in glomeruli of rats with anti-GBM nephritis (group II) was also significantly reduced compared to saline injected rats (19.8 ± 6.1 units, $P < 0.005$). In contrast, no reduction in glomerular ADPase activity was observed in anti-FX1A injected rats (group III; 61.5 ± 11.0 units) as compared to control animals (group IV).

As can be seen in Figure 5, treatment of rats with SOD and catalase after injection of anti-Thy1 IgG resulted in significantly more DAB staining compared to rats receiving saline after the anti-Thy1 injection (29.9 ± 10.7 units, $P < 0.05$). Similarly, the decrease in stainability for ADPase in anti-GBM injected rats (group II) could also be significantly reduced by treatment of the animals with scavengers (34.5 ± 3.6 units) as compared to rats receiving saline instead of scavengers (Fig. 5, $P < 0.025$).

Platelet aggregation

Platelet aggregation, immunohistochemically demonstrated with Moab pl-I (Fig. 6), was increased in glomeruli of rats with anti-Thy1 (group I) and anti-GBM nephritis (group II), whereas in rats with anti-FX1A nephritis (group III) or in saline injected control rats (group IV) no intraglomerular platelet aggregation could be detected 24 hours after injection of nephritogenic

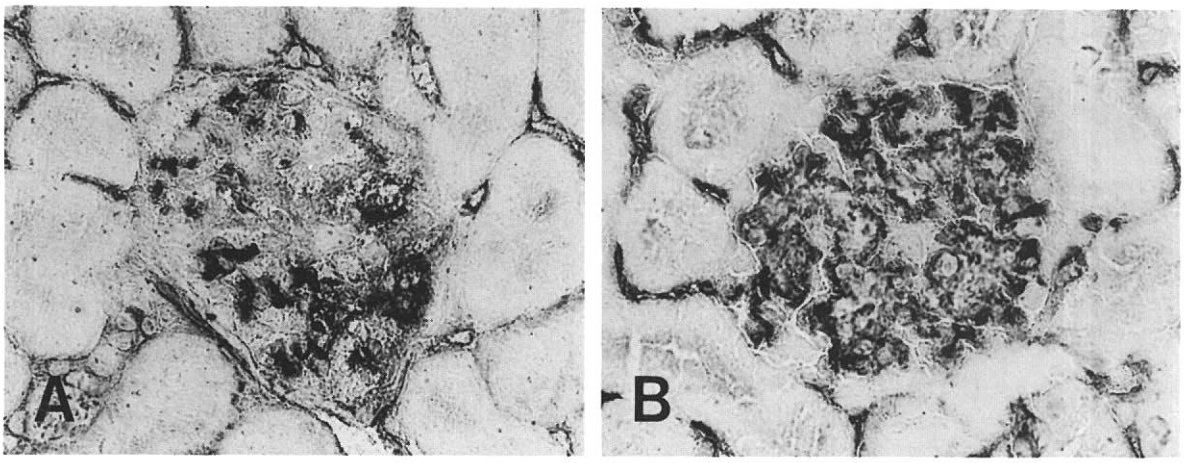

Fig. 3. *Representative glomeruli in cryostat sections from kidney of rats 24 hr after induction of* **(A)** *anti-Thy1 nephritis (group I) versus* **(B)** *saline injected control rats (group IV) staining for ADPase using the cerium based method at the light microscopical level.* Note decreased amount of reaction product in glomerulus of a nephritic rat (A) as compared to control rat (B) (original magnification ×350).

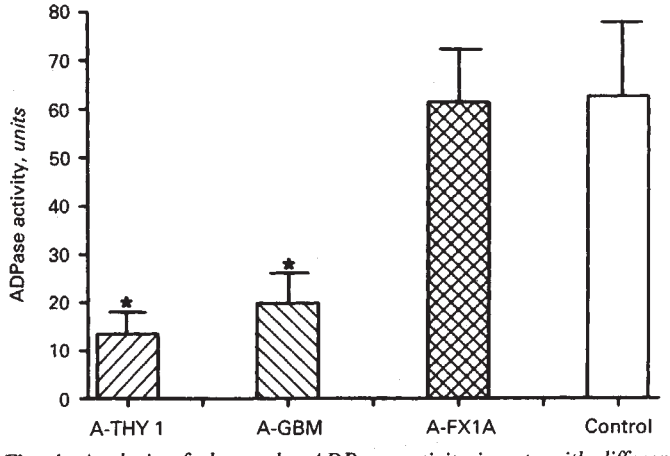

Fig. 4. *Analysis of glomerular ADPase activity in rats with different forms of experimental GN (anti-Thy1, anti-GBM, anti-FX1A) or untreated control rats using computerized image analysis.* Columns represent arithmetic means (± SD) of ADPase activity expressed in units (optical density per standard glomerular area) in 6 rats per group. Note reduced staining in kidney of rats with acute glomerulonephritis (hatched columns) in contrast to rats with anti-FX1A nephropathy (group III, crossed columns) as compared to control rats (group IV, open columns). Statistical significance as indicated. * = $P < 0.005$ (Wilcoxon). Abbreviations as indicated in Fig. 2.

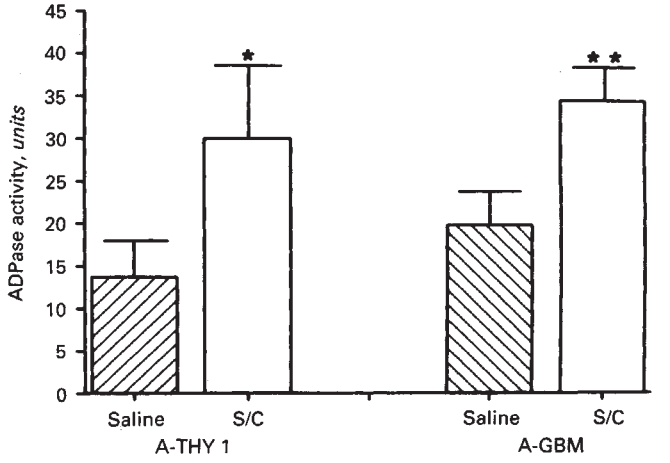

Fig. 5. *Analysis of ADPase activity in glomeruli of nephritic rats (anti-Thy1 and anti-GBM) treated with either saline or SOD and catalase (S/C).* Columns represent mean optical density per standard glomerular area (± SD) expressed in units in 6 rats per group. A clear increase in glomerular ADPase activity can be observed in rats treated with S/C (open columns) compared to rats treated with saline (hatched columns). Statistical significance as indicated. * = $P < 0.05$; ** = $P < 0.025$ (Wilcoxon). Abbreviations as indicated in Fig. 2.

antibody or saline. In Figure 7 quantitative analysis of these observations is depicted, showing a significant increase of the mean glomerular area stained for aggregated platelets in anti-Thy1 injected rats (group I) as compared to rats of group IV (7.0% ± 1.7 vs. 0.2% ± 0.1 platelet aggregation/glomerulus, $P < 0.005$). Also, in glomeruli of rats with anti-GBM nephritis (group II) a significant increase compared to control rats (group IV) was seen (4.7% ± 2.9 vs. 0.2% ± 0.1 platelet aggregation/glomerulus, $P < 0.005$). In contrast, no significant increase in degree of pl-1 staining was observed in rats with anti-FX1A nephritis (0.7% ± 0.5 platelet aggregation/glomerulus).

As shown in Figure 8, after treatment with scavengers a significant reduction in stainability for intraglomerular platelets was seen in rats with anti-Thy1 and anti-GBM GN (3.6 ± 1.3% and 2.7 ± 0.1% platelet aggregation/glomerulus, respectively, $P < 0.05$) compared to nephritic rats receiving treatment with saline.

In Figure 9 the logarithm of glomerular ADPase activity observed in the different experimental and control groups, is compared with the intraglomerular platelet accumulation in these animals. Gradually decreasing ADPase activity in the various experimental groups was observed, as depicted in relationship with the observed platelet aggregation in these groups ($r = 0.9884$).

Ex vivo perfusion studies with inflammatory cells

To study whether activated inflammatory cells can directly affect glomerular ADPase activity and subsequently influence intraglomerular thrombotic tendency, ex vivo perfusion studies with PEC and platelet rich plasma were performed. Immedi-

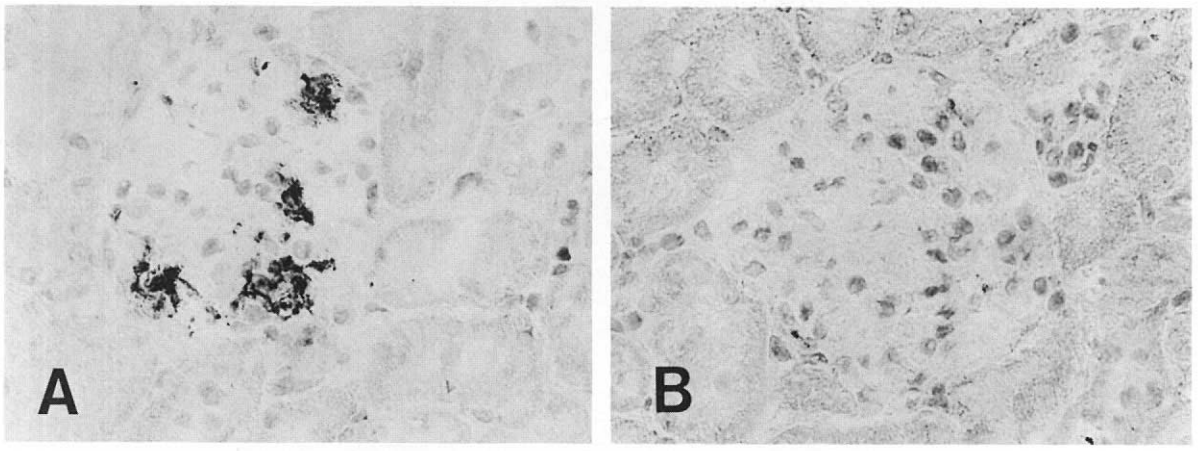

Fig. 6. *Representative glomeruli from kidney sections of* **(A)** *anti-Thy1 injected rats (group I) and* **(B)** *saline injected rats (groups IV) stained for rat platelets using a mouse biotinilated monoclonal antibody against rat platelets (pl-1) and DAB as a second step.* Platelet aggregates can be demonstrated 24 hr after induction of anti-Thy1 nephritis (A) but not in control animals (B) (original magnification ×350).

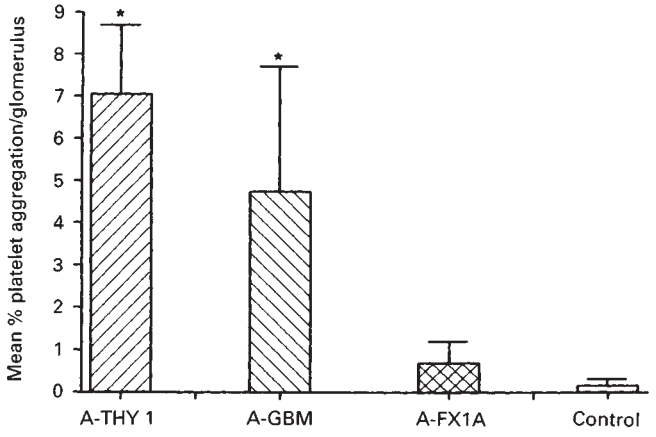

Fig. 7. *Analysis of intraglomerular platelet aggregation using image analyzing techniques in rats with different forms of GN (anti-Thy1, anti-GBM and anti-FX1A) or saline injected control animals.* Results are expressed as arithmetic means of percentages glomerular areas (± SD) stained with pl-1 in 6 rats per group. Note the considerable increase of aggregation in the inflammatory forms of glomerulonephritis (hatched columns) in contrast to anti-FX1A glomerulopathy as compared to control animals (open columns). Statistical significance as indicated. * = $P < 0.005$ (Wilcoxon). Abbreviations as in Fig. 2.

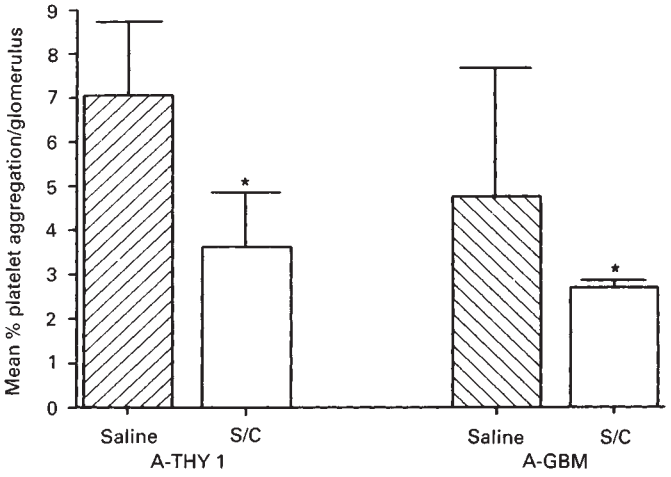

Fig. 8. *Analysis of intraglomerular platelet aggregation in nephritic rats treated with saline (N = 6) or SOD and catalase (N = 6).* Columns represent mean percentage of glomerular area (± SD) stained for platelets using biotinilated pl-1. Treatment with SOD and catalase (open columns) significantly reduces platelet aggregation compared to saline treatment (hatched columns) in rats with anti-Thy1 (left set of columns) as well as in rats with anti-GBM glomerulonephritis (right set of columns). Statistical significance as indicated. * = $P < 0.05$ (Wilcoxon). Abbreviations as indicated in Fig. 2.

ately following PEC perfusion a biopsy was taken and processed for ADPase evaluation. Subsequently alternate perfusion with platelets and ADP was carried out.

As can be deduced from Figure 10, glomerular ADPase activity of untreated kidneys was reduced following ex vivo perfusion with inflammatory cell suspensions compared to control perfusions with buffer supplemented with SOD. In addition, this decrease in glomerular ADPase activity could be inhibited a great deal when SOD was added to the PEC suspension.

As shown in the right set of columns in Figure 10, impairment of ADPase by inflammatory cell perfusion ex vivo is associated with increased stainability for human fibrinogen following alternate perfusion with human PRP and ADP compared to control perfusions with buffer supplemented with SOD. In contrast, kidneys perfused with PEC supplemented with SOD showed less staining for human fibrinogen compared to kidneys perfused with PEC. Catalase supplementation to the PEC suspension did not influence the effect of PEC upon glomerular ADPase or platelet aggregation ex vivo (results not shown).

Discussion

In the present study, influx of oxygen free radical-producing cells was evaluated in three models of experimental glomerulonephritis and related to intraglomerular platelet aggregation as well as glomerular ADPase activity. It has been shown that the

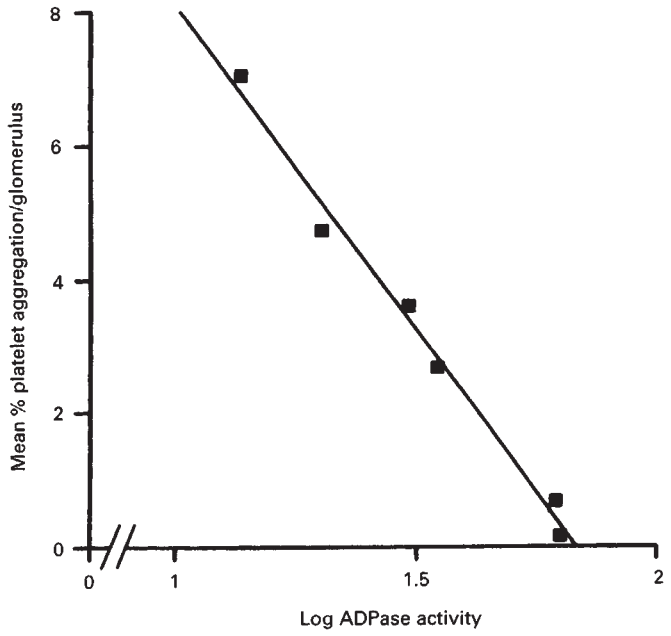

Fig. 9. *Relationship between glomerular ADPase activity and intraglomerular platelet aggregation in the different experimental groups as determined by computerized image analysis.* ADPase activity is measured as optical density per standard glomerular area in units and calculated in a log scale. Statistical significance (regression analysis) as indicated ($r = 0.9884$).

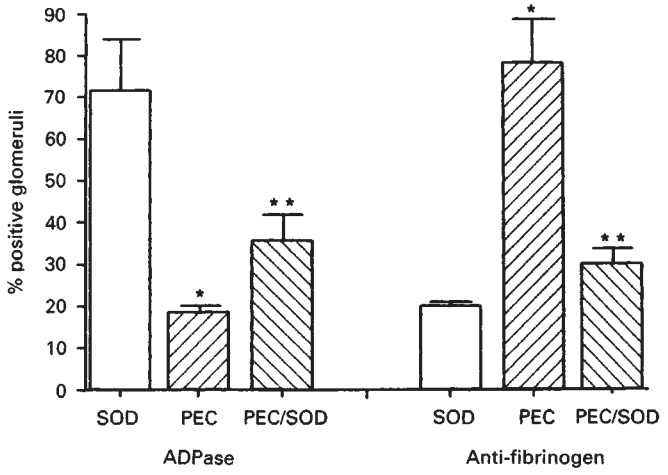

Fig. 10. *Mean percentage of glomeruli with normal ADPase activity (left set of columns) and mean percentage of glomeruli positive for human fibrinogen (right set of columns) were semiquantitatively scored in the experimental groups V, VI and VII (5 rats per group).* PEC perfusion reduces the number of glomeruli with normal ADPase activity and increases glomerular fibrinogen staining as compared to control perfusions (SOD; $*P < 0.005$), whereas both effects of PEC can significantly be inhibited by SOD addition to the PEC suspension compared to perfusion with PEC alone ($**P < 0.005$). Statistical significance as indicated (Wilcoxon).

presence of glomerular ADPase activity in the rat kidney, which is independent of proteinuria [34], is related to intraglomerular thrombotic tendency [13].

The involvement of neutrophils in the early phase of the three forms of GN studied in the present paper has been well documented. Thus, in anti-GBM nephritis in the rat within three hours influx PMN can be observed in glomeruli [5] and in anti-Thy1 nephritis accumulation of PMN in glomeruli has also been described [4]. In contrast, in the early (heterologous) phase of anti-FX1A nephritis inflammatory cells seem not to play a major role [18, 19]. As is shown in Figures 1 and 2 these observations could be confirmed in the present study using histochemical staining methods for oxygen free radicals, that is, O_2^- and H_2O_2 produced by these cells in situ [13]. Specificity of free radical staining was routinely checked by inhibition studies with SOD and catalase in vitro. By morphological examination these cells appeared to be predominantly polymorphonuclear cells with minor presence of monocytes. As can be seen in Figure 2, SOD and catalase treatment in vivo did not influence the number of activated neutrophils in glomeruli of rats with anti-Thy1 and anti-GBM nephritis. Therefore it is highly unlikely that treatment of rats with scavengers influences intraglomerular antibody deposition a great deal, nor does it significantly interfere with other parts of the effector phase of the inflammatory response. The observation that treatment of the diseased animals with scavengers does not result in reduced staining for O_2^- and H_2O_2 in the kidney sections probably relates to the relative short t½ of SOD and catalase (6 min and 3 to 5 min, respectively) [35], and the four hour time interval between treatment and sacrifice.

In addition to O_2^- producing cells, glomerular ADPase activity was demonstrated using the cerium based method at the light microscopical level. This method clearly demonstrates differences in ADPase activity (Fig. 3). As can be seen in Figure 4, the increase in O_2^- producing cells occurred concomitantly with a decrease in glomerular ADPase activity exclusively in rats with anti-GBM and anti-Thy1 nephritis. Moreover, platelet aggregation detected with pl-1 (Fig. 6) could also be demonstrated exclusively in glomeruli of rats with anti-Thy1 and anti-GBM nephritis (Fig. 7). The striking relationship between inflammatory cell influx on one hand and decreased glomerular ADPase activity in association with intraglomerular platelet aggregation on the other hand is in line with previous observations, suggesting that oxygen free radicals play a major role in inactivation of glomerular ADPase [15, 16, 36]. The significant protective effect of oxygen free radical scavengers administered in vivo upon glomerular ADPase activity (Fig. 5) and platelet aggregation (Fig. 8) in rats with anti-GBM as well as in rats with anti-Thy1 nephritis also supports this notion. The relative short half-life of SOD and catalase [35] may account for the fact that in spite of the intensive scavenger treatment, some ADPase impairment and platelet aggregation is still observed in these models. Taken together, however, the straightforward relationship between glomerular ADPase activity and platelet aggregation in vivo (Fig. 9) demonstrates the importance of glomerular ADPase as an antithrombotic principle within the rat kidney.

To investigate whether oxygen free radical-producing inflammatory cells are able to influence glomerular ADPase activity directly and induce an alteration in glomerular thrombotic tendency, we also studied the effect O_2^- producing cell suspensions (that is, PEC) perfused ex vivo in kidneys of normal rats. Platelet aggregation was detected by immunochemical demonstration of in situ fibrinogen deposition. It is shown by other authors that platelets may expose fibrinogen receptors upon

activation leading to binding of fibrinogen [32, 33]. As can be seen from Figure 10, kidney perfusion with PEC suspensions containing predominantly PMN was able to affect glomerular ADPase activity in an O_2^- dependent manner. The demonstration that subsequent alternate perfusion with platelets and ADP in kidneys pre-perfused with activated PEC facilitated intraglomerular platelet aggregation, which is also inhibitable by SOD supports the proposed sequence of events: oxygen free radicals produced by PMN affect ADPase activity, which leads to facilitation of intraglomerular platelet aggregation.

However, comparison of in vivo and ex vivo results should be taken with caution since inflammatory cells in both conditions may differ in many ways. This is illustrated by the fact that in vivo the combination of SOD and catalase was necessary to protect glomerular ADPase activity and inhibit platelet aggregation, whereas in the ex vivo perfusion studies, SOD alone was sufficient to inhibit the action of toxic oxygen products. The necessity of both scavengers in vivo suggests that in addition to O_2^-, H_2O_2 also plays a role in ADPase impairment. This H_2O_2 is generated by conversion of O_2 , either spontaneously or catalyzed by SOD [37], whereas H_2O_2 in association with myeloperoxidase (MPO) activity of neutrophils has been shown to induce intraglomerular damage in the rat kidney [38]. Both O_2^- production and the MPO-halide-H_2O_2 system are histochemically demonstrated in vivo during anti-Thy1 and anti-GBM nephritis (Figs. 1 and 2). The fact that SOD alone was effective during the ex vivo perfusion studies suggests that the MPO-halide-H_2O_2 system is not involved in these conditions, which may be related to the mode of activation of neutrophils in these experiments.

The demonstration of increased platelet aggregation in rats with anti-Thy1 and anti-GBM nephritis (Fig. 7) is in agreement with observations of many authors of increased platelet aggregation during the acute phase of glomerulonephritis [1, 2]. This platelet aggregation can be initiated in several ways during glomerulonephritis. For instance, immune complexes are able to activate platelets by their Fc receptors [9], or may stimulate production of platelet activating factor (PAF) by infiltrating cells [10] or TXA_2 by intrinsic glomerular cells [8]. Whatever agonist may be involved in the initiation of platelet aggregation in the present experimental glomerulopathies, if the GBM associated ADPase activity is protected, its antithrombotic activity will prevent intraglomerular thrombosis. Thus, as can be seen from Figures 2 and 8, while the number of activated PMN's is not affected by SOD and catalase treatment (proaggragatory stimuli from these cells are still present), platelet aggregation is significantly reduced in rats with anti-Thy1 or anti-GBM nephritis after treatment with these oxygen scavengers.

The observed reduction of ADPase activity by neutrophils and subsequent inhibition of platelet aggregation by scavengers may be an explanation for the benefical effects of scavengers upon tissue damage after induction of nephrotoxic serum nephritis [5, 6] reported in other studies. Platelet aggregation in the microcirculation can have damaging effects [39, 40] by obstruction of capillaries and release of vasoactive amines, cationic proteins or chemotactic factors for neutrophils [reviewed in 9]. Interestingly, it has also been reported that ATP and ADP release by activated platelets potentiates O_2^- production by PMN [41–43]. The beneficial effects of platelet depletion in the arthus reaction [44] and experimental pneumonitis [45] illustrates the relation between platelet aggregation and the inflammatory process. A powerful antithrombotic mechanism within glomeruli may therefore be important, especially since blood cells are, through the fenestrated endothelium, in close contact with the GBM. This GBM consists predominantly of collagen, a stimulator of platelet aggregation [46]. ADPase activity within the GBM may therefore, in addition to heparan sulphate proteoglycanes and endothelial prostacyclin production, be of major importance as an antithrombotic mechanism within the glomerulus of the kidney.

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